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Purification of a bifunctional amylase/protease inhibitor from ragi (*Eleusine coracana*) by chromatography and its use as an affinity ligand

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ABSTRACT

An ammonium sulphate fraction (20–60%) of bifunctional amylase/protease inhibitor from ragi (*Eleusine coracana*) was purified by affinity chromatography to give 6.59-fold purity with 81.48% yield. The same ammonium sulphate fraction was also subjected to ion exchange chromatography and was purified 4.28-fold with 75.95% yield. The ion exchange fraction was subjected to gel filtration and the inhibitor was purified to 6.67-fold with 67.36% yield. Further sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to check the homogeneity of purified amylase/trypsin inhibitor obtained through affinity, ion exchange and gel chromatography. The molecular weight of the inhibitor was found to be 14 kDa. This purified inhibitor was used as affinity ligand for the purification of a commercial preparation of pancreatic amylase.

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1. Introduction

The amylase inhibitors are widely distributed in higher plants and microorganisms. In case of higher plants, many kinds of amylase inhibitors have been purified, mainly from cereals and legume seeds. Research on plant amylase inhibitors for most part has been concerned with cereals plants because of the importance of cereals as a rich source of protein for animal and human consumption. The inhibitors are widely distributed among cereal grains such as ragi, rye, kidney bean, barley, maize and pea [1]. Some of the amylase inhibitors are produced by microorganism such as *Streptomyces diastaticus* [2], *Streptomyces tendae*, *Streptomyces aurofaciens* [3].

Plant proteins that inhibit various types of enzymes have been extensively studied. The most commonly occurring inhibitors are the proteinase and amylase inhibitors. α-Amylase inhibitors, which are proteinaceous in nature, are widely distributed in the plant kingdom [4]. These inhibitors have been isolated and purified from several sources, particularly from cereals.

Cereal amylase inhibitors are mostly double headed in nature. Double-headed inhibitors often show two different binding sites which are specific to different proteases. However, in some cases, especially from cereals, bifunctional protease/α-amylase inhibitors have also been reported [5,6]. These inhibitors have specific action against mammalian and insect amylase and trypsin enzyme. Cereal inhibitors have generally low molecular weight (10,000–50,000). These inhibitors have natural role in the control of endogenous α-amylase activity or in the defense against pathogen and pests [7]. Amylase/trypsin inhibitors are reported to be anti-nutritional factor and have therapeutic application [8]. Apart from this defense mechanism these bifunctional inhibitors are potentially valuable 'two-in-one' affinity ligands for the purification of proteases and α-amylases.

Lately there has been considerable effort in developing affinity-based efficient bioseparation protocols for enzymes/proteins with fewer steps [9,10]. A critical factor is the cost of the affinity ligands. Hence efficient purification procedure for a double-headed inhibitor serves two purposes: (a) reduces the cost of the affinity ligand; (b) twin uses of the same affinity media mean greater convenience as well as further economy.

In the present work, the ammonium sulphate fraction (20–60%) from ragi was taken for further purification by affinity chromatography. Attempts were also carried out to purify amylase/trypsin inhibitor from ragi by ion exchange chromatography on cation exchanger. This was followed by gel filtration [11]. The purified amylase/trypsin inhibitor was further used as affinity ligand for the purification of a commercial preparation of pancreatic amylase [12].

2. Materials and methods

2.1. Materials

Ragi seeds (*Eleusine coracana*) were purchased from local market. Seralose 6B was procured from Sisco Research Lab, Mumbai,

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India. Pancreatic amylase (12.5 U/ml) was procured as a gift sample from Biocon India Ltd., Bangalore, India. Trypsin (450 U/mg) was procured as gift sample from Advanced Biochemical Ltd., Thane, Mumbai. Sulphopropyl cation exchanger (ECONO-PAC High S) pre-packed column was purchased from Bio-Rad Ltd., India. Sephadex G-50 was purchased from Amersham Bioscience Ltd., Chennai, India. Disodium hydrogen phosphate, sodium dihydrogen phosphates and enzyme grade ammonium sulphate, acrylamide, N,N-bisacrylamide, glycerol, glycine, ammonium persulphate and tetra methylene ethyl diamine (TEMED) were purchased from Sisco Research Lab, Mumbai. India. Molecular marker was purchased from Banglore Genei Pvt. Ltd. Tris buffer AR, copper sulphate pentahydrate, sodium potassium tartarate AR, Folin-Ciocalteu phenol reagents were purchased from E Merck (India) Ltd. All other chemicals used were of analytical grade.

2.2. Methods

2.2.1. Estimation of amylase inhibitor/trypsin inhibitor activity and protein content

The amylase inhibitor activity was calculated using starch as the substrate [13]. The assay was carried out at 37 °C. One unit of amylase inhibitor was defined as that amount, which inhibits the activity of porcine pancreatic amylase by 1 U under the assay conditions [14]. Trypsin inhibitor activity of the crude ragi extract as well as various fractions obtained during the course of purification was estimated using casein as the substrate [15]. One trypsin inhibitor unit was equivalent to decrease in absorbance by 0.01 units at 280 nm.

Protein content of inhibitor rich fractions was determined by the Folin–Lowry method [16] using bovine serum albumin as the standard protein. Fold purity and recovery yield (% yield) were calculated as mentioned below:

$$\text{Fold purity (FP)} = \frac{\text{Specific activity after purification}}{\text{Specific activity before purification}}$$

$$\text{Recovery (\% yield)} = \frac{\text{Total activity after purification}}{\text{Total activity before purification}} \times 100$$

2.2.2. Preparation of crude inhibitor extract of ragi (E. coracana)

Preparation of crude inhibitor extract of ragi was carried out using the method followed by Saxena et al. [17].

2.2.3. Purification of amylase/protease inhibitor

2.2.3.1. Ammonium sulphate precipitation. Ammonium sulphate precipitation was carried out using the method followed by Saxena et al. [17] and the 20–60% fraction was used for further purification studies.

2.2.3.2. Affinity chromatography. Seralose 6B, agarose-based matrix was activated with Cyanogen Bromide (CNBr) using the method followed by Hermanson et al. [18]. The gel was then washed with ice-cold water and ice-cold coupling buffer (0.1 M $NaHCO_3$, pH 8.5). 20 ml of this CNBr activated matrix was suspended in equal volume of coupling buffer (0.1 M $NaHCO_3$, pH 8.5), which contained 2% (w/v) of trypsin ligand. The gel was stirred at 4 °C, 24 h and then washed several times with coupling buffer (0.1 M $NaHCO_3$, pH 8.5), 1 M NaCl and water to remove unreacted ligand. The excess active site on the gel was blocked by suspending it in 1 M ethanolamine (pH 9, 1 h) and then packed in a column (Pharmacia LKB 10 cm, internal diameter 1.0 cm; bed volume 3 ml) with equilibrating buffer (0.02 M phosphate buffer, pH 6.9, 30 ± 2 °C). The column was equilibrated with 5 column volumes of binding buffer (0.02 M phosphate buffer, pH 4.0) at a linear flow rate of 0.693 cm/min. After loading of 20–60% ammonium sulphate fraction, the column was washed using same equilibration buffer (0.02 M phosphate buffer, pH 6.9, linear flow rate of 0.693 cm/min) till the effluent was free from protein. After washing, elution was carried out using a mixture of 50 mM HCl and 20 mM NaCl (pH 3.0). The effluent fractions (0.5 ml, 20 fractions, isocratic elution) were collected using a fraction collector (Bio-Rad Model 2110). Enzyme inhibitor activities (U/ml) of all the fractions were analyzed using the aforementioned methods. The effluent protein concentration was detected at 280 nm and the elution chromatogram was plotted [11].

2.2.3.2.1. Batch study to determine adsorption of amylase/trypsin inhibitor on affinity matrix. Adsorption isotherm, required in order to determine adsorption capacity of adsorbent for amylase/trypsin inhibitor, was determined as follows.

Dilutions of ammonium sulphate fraction (20–60%) containing different concentrations of amylase inhibitor (0.095–11.44 U/ml) and trypsin inhibitor (3.3–400 U/ml) was loaded in stoppered tubes containing 1 ml of pre-equilibrated matrix (0.02 M phosphate buffer, pH 6.9, 30 ± 2 °C). The tubes containing loaded amylase/trypsin inhibitor fraction was kept for equilibration on a rocker shaker, till the supernatant equilibrium concentration of protein and enzyme activity was reached. 0.1 ml sample was removed from supernatant and assayed for enzyme inhibitor activity as described in Section 2.2.1. A graph was plotted between adsorbed enzyme inhibitor activity (q^*) versus corresponding supernatant equilibrium concentration (C^*). The plots indicate the nature of adsorption. The amount of enzyme bound to the adsorbent q^* was calculated as the difference between total amount of enzyme units loaded and units present in the supernatant after 2 h of equilibration. Maximum adsorption capacity $q^{\max}$ was determined from the plot of q^* versus C^* and the type of isotherm was found by plotting a graph of $1/q^*$ versus $1/C^*$. Dissociation constant k_d between enzyme and ligand was determined as follows:

Adsorption isotherm equation:

$$q^* = \frac{q_m C^*}{k_d + C^*} \tag{1}$$

where k_d = dissociation constant of the equilibrium reaction.

Rearranging Eq. (1) we get

$$\frac{C^*}{q^*} = \frac{C^*}{q_m} + \frac{k_d}{q_m} \tag{2}$$

Value of k_d can be determined from straight-line plot of C^*/q^* against C^*. The intercept of such plots on the C^* axis is at $-k_d$.

2.2.3.3. Purification of amylase/trypsin inhibitor by ion exchange chromatography. Purification was carried out using pre-packed Bio-Rad sulphopropyl cation exchanger (ECONO-PAC High S) matrix [19] having a column diameter of 10 mm and bed volume of 3 ml. The column was equilibrated using 4 column volumes of 0.02 M phosphate buffer, pH 6.9. 4 ml of ammonium sulphate purified enzyme inhibitor sample was loaded onto this column and linear flow rate was maintained at 0.693 cm/min. This was followed by washing the column with the equilibrating buffer (0.02 M phosphate buffer, pH 6.9) to remove unbound or weakly bound protein. Elution (0.5 ml, 16 fractions, gradient elution) was done by increasing the ionic strength of buffer (0.02 M phosphate buffer, pH 6.9) stepwise from 0.1 to 1 M using NaCl. Protein eluted in each step was checked for amylase/trypsin inhibitor activity [20].

2.2.3.4. Purification of amylase/trypsin inhibitor by gel permeation chromatography on Sephadex G-50. The partially purified ion exchange fraction (fractions 3–15) was further purified by gel chromatography. Sephadex G-50 was selected as the matrix [20,21]. 5 g of Sephadex G-50 was kept for swelling in 80 ml of 0.02 M phosphate buffer, pH 6.9. After 6 h the gel was deaerated by applying

vacuum and resuspended in fresh buffer (0.02 M phosphate buffer, pH 6.9). Around 5 ml of buffer (0.02 M phosphate buffer, pH 6.9) was filled into a column (bed height 15 ml) and the equilibrated gel was slowly added. 4 ml sample (ion exchange purified) was then loaded on the column and followed by elution using 0.15 M phosphate buffer, pH 6.9, having a linear flow rate of 0.693 cm/min. The fraction sizes were approximately 0.5 ml (13 fractions) and were analyzed for amylase/trypsin inhibitor activity.

2.2.4. Electrophoresis and molecular mass determination of purified amylase/protease inhibitor

SDS-PAGE electrophoresis was performed to check the homogeneity of amylase/trypsin inhibitor purified through packed bed affinity, ion exchange and gel chromatography. It was carried out using 15% gel as per the procedure stated in the literature [22] on a Thermo Electron Corporation gel electrophoresis unit and was compared with standard molecular weight markers.

2.2.5. Immobilization of amylase inhibitor on Seralose 6B matrix

Amylase inhibitor from ragi purified using affinity chromatography was used as ligand sample. 2 ml of CNBr activated Seralose 6B (using method described in Section 2.2.3.2) was suspended in 10 ml of amylase inhibitor solution (1.41 U/ml) and kept in an incubator shaker (100 rpm, 4 °C) for 8 h. 0.5 ml supernatant was removed after every 2 h and checked for amylase inhibitor activity. The Seralose 6B beads immobilized with amylase inhibitor were then washed with 0.02 M phosphate buffer (pH 6.9). 2 ml of 1 M ethanolamine of pH 9.0 was added to block the active cyanate ester functional groups. The reaction was allowed to take place for 1 h. The beads were then washed with distilled water to remove ethanolamine.

2.2.6. Interaction of immobilized amylase inhibitor and pancreatic amylase

0.1 g of commercial pancreatic amylase (pancreatin) was dissolved in 100 ml of 0.02 M phosphate buffer, pH 6.9. The Seralose 6B beads containing immobilized amylase inhibitor were suspended in 2 ml of pancreatin solution. It was then kept in incubator shaker at 100 rpm and 4 °C for 90 min. 0.5 ml of supernatant was removed after 30, 45, 60 and 90 min and checked for amylase activity by DNSA method at 540 nm [13].

2.2.6.1. Definition of amylase unit. One unit of amylase enzyme is defined as the amount of enzyme that liberates 1 μmole of reducing sugar (calculated as glucose) per min at 37 °C under assay conditions [23].

All the experiments were performed in triplicates and the difference in the readings was less than or equal to ±3%.

3. Results and discussion

3.1. Affinity chromatography

Equilibrium adsorption isotherm was studied to find out the type of isotherm pattern followed by the affinity system developed for the purification of amylase/trypsin inhibitor. The enzyme inhibitor followed a typical Langmuir type of isotherm as seen in Fig. 1. There is a possibility that the enzyme inhibitor is interacting with the ligand through one of its active site, since amylase/trypsin inhibitor in sample solution binds with the trypsin ligands at its N-terminal site. It clearly shows that amylase inhibitor active site is free for taking part in the reaction. The amylase/trypsin inhibitor activity showed a sharp breakthrough pattern (Fig. 2). This indicates a good performance of the affinity matrix. The reason behind these observations may be that either the ligand could be having strong affinity for the enzyme inhibitor or the linear flow rate (0.693 cm/min) is very low [24]. Also the dissociation constant (k_d) value (determined by putting value from plot $1/q^*$ versus $1/C^*$ in the Langmuir adsorption isotherm) was found to be 1.17×10^{-5} M which is within the range with the findings of Scopes [19] who observed that k_d value should be between 10^{-4} and 10^{-8} for better adsorption and elution of the proteins.

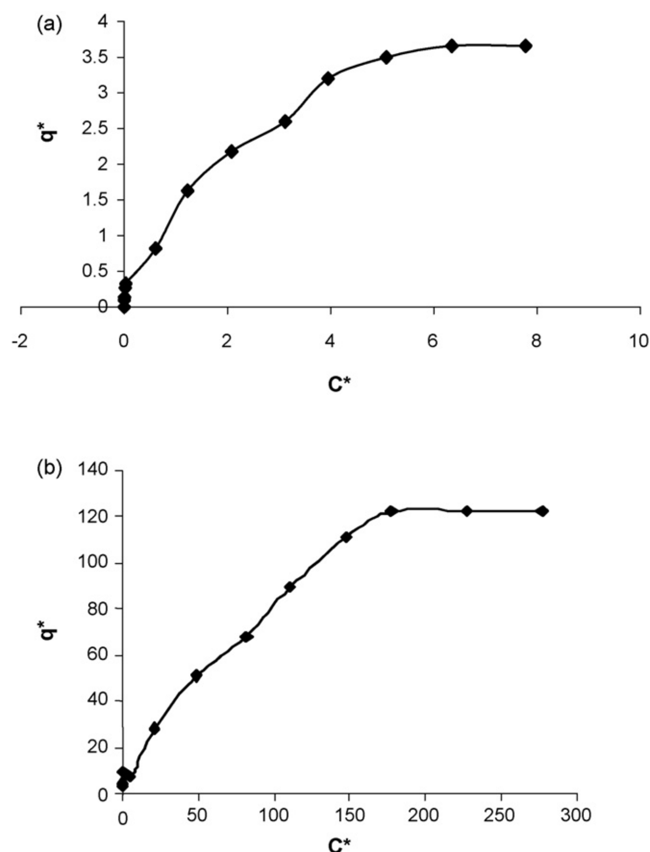

Fig. 1. Equilibrium adsorption isotherm for the (a) adsorption of amylase inhibitor activity from ragi (*Eleusine coracana*) on trypsin coupled affinity ligand and (b) adsorption of trypsin inhibitor activity from ragi (*Eleusine coracana*) on trypsin coupled affinity ligand. Where q^* is adsorbed enzyme inhibitor activity (U/ml) and C^* is corresponding enzyme inhibitor activity in supernatant.

3.1.1. Elution chromatogram of amylase/trypsin inhibitor

Elution chromatogram is as shown in Fig. 3. The eluted fraction was analyzed with respect to amylase/trypsin inhibitor activity (U/ml). The peaks recorded were symmetrical. It was observed that

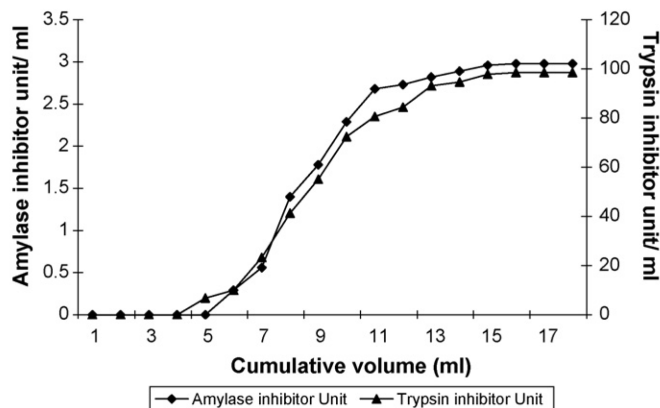

Fig. 2. Breakthrough curve of amylase/trypsin inhibitor adsorption obtained from ragi (*Eleusine coracana*) on trypsin coupled affinity ligand.

Table 1
Overall purification of amylase/trypsin inhibitor from ragi (*Eleusine coracana*).

Step	Vol (ml)	Inhibitor	Unit/ml	Total activity (unit)	Protein content (mg/ml)	Total protein content (mg)	Specific activity (U/mg)	F.P	% Yield of inhibitor
Ammonium sulphate fraction (20–60%)	12	AIU TIU	3 100	36.3 1200	0.6	7	5.1 182	1 1	100 100
Affinity chromatography	21	AIU TIU	1.4 41	30 859	0.04	1	33.6 974.4	7 5.3	82 72
Ion exchange chromatography	24	AIU TIU	1.2 42	28 1002	0.05	1.3	22 789	4.3 4.3	76 84
IEC + gel permeation chromatography	120	AIU TIU	0.2 5	25 604	0.01	1	34 838.3	7 5	67 50

AIU, amylase inhibitory unit; TIU, trypsin inhibitory unit. The above experiments were carried out in triplicates and individual (in terms of specific activity and % yield) readings varied in the range of ±3%.

both amylase and trypsin inhibitor activities were found in similar fractions; even the affinity purification did not separate the bifunctional inhibitors. Similar findings have also been reported by Shivaraj and Pattabiraman [20].

Recovery of 82 and 72% and purification fold of 7 and 5.3 of amylase inhibitor and trypsin inhibitor respectively was obtained as given in Table 1.

3.2. Ion exchange chromatography

Fig. 4 shows the elution chromatogram of amylase/trypsin inhibitor during purification by ion exchange chromatography. It was observed that between fractions 5 and 15 there was a considerable increase in inhibitor activity.

Binding of the amylase/trypsin inhibitor was carried out at pH 6.9. This was because isoelectric point for the enzyme inhibitor was reported to be more than 10 [25]. Thus at pH 6.9 the enzyme would be positively charged. As a result of this it could be exchanged with the counter ions of sulphopropyl cationic resin. It was observed that the enzyme inhibitor could be eluted using 0.1–1 M NaCl. The chromatographic fractions thus obtained were checked for both amylase and trypsin inhibitor activities. Using this method amylase inhibitor was purified 4.3-fold with 76% yield and trypsin inhibitor was purified 4.3-fold with 84% yield (Table 1). It was however observed that ion exchange chromatography showed lesser purification as compared to affinity chromatography. This may be because in ion exchange chromatography separation is based on charge of the biomolecules and as seen in Fig. 4 even the unwanted proteins have the same net charge, as all of them were eluted out in the same fraction along with the amylase/trypsin inhibitor. This must have contributed to the low fold purification. It was also observed that the amylase and trypsin inhibitor activities were associated with each other, i.e. the same protein seemed to have active site for both the enzyme inhibitors.

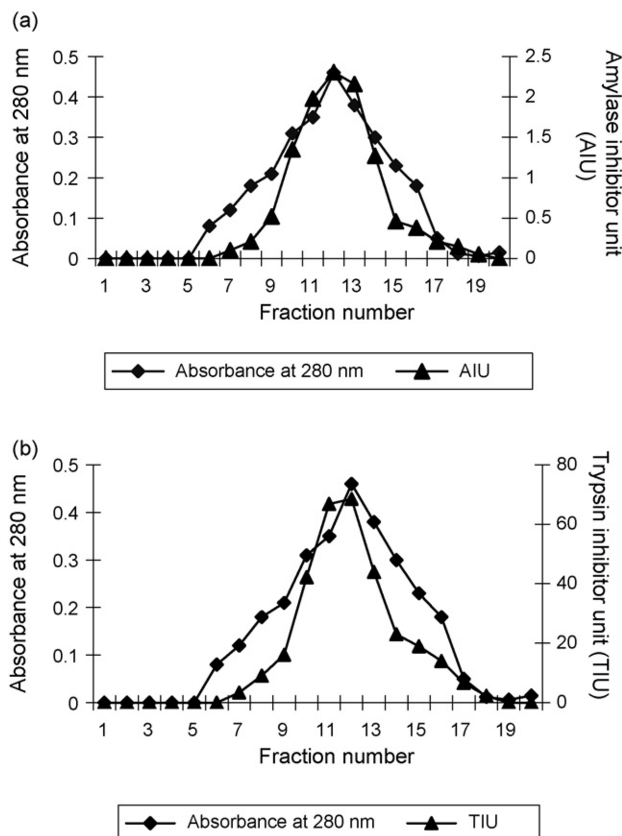

Fig. 3. Elution chromatogram of amylase/trypsin inhibitor from affinity chromatography for (a) amylase inhibitor and (b) trypsin inhibitor.

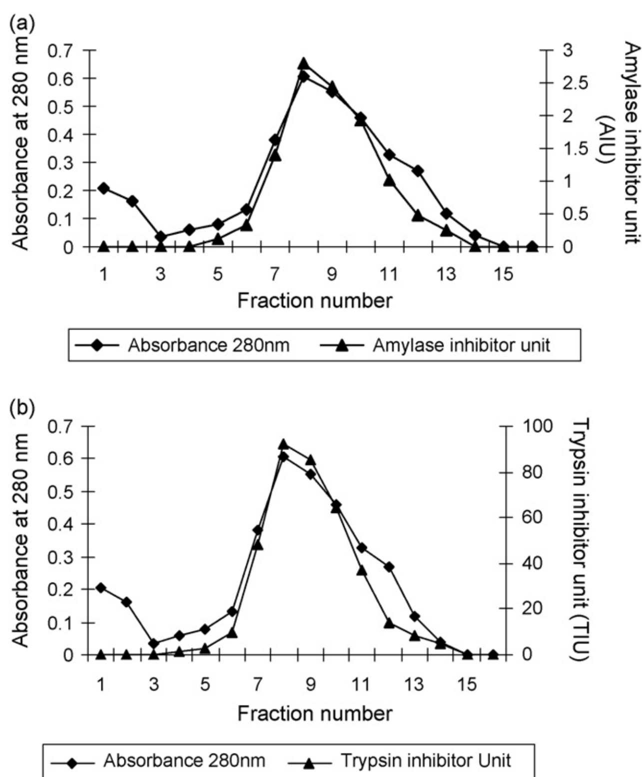

Fig. 4. Elution chromatogram of amylase/trypsin inhibitor by ion exchange chromatography for (a) amylase inhibitor and (b) trypsin inhibitor.

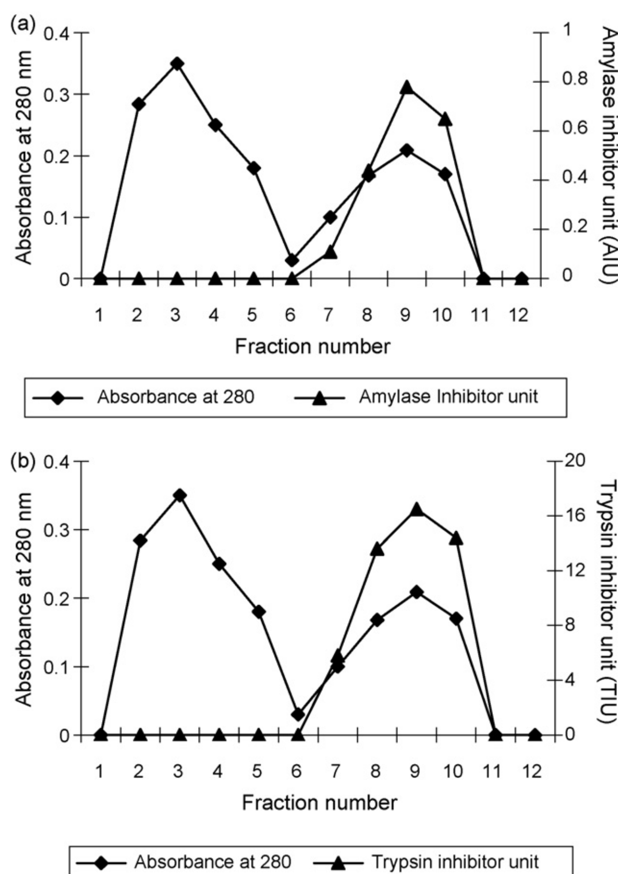

Fig. 5. Elution chromatogram of amylase/trypsin inhibitor by gel filtration using Sephadex G-50 for (a) amylase inhibitor and (b) trypsin inhibitor.

3.3. Gel permeation chromatography

Gel permeation chromatography was chosen for purification after ion exchange chromatography as the latter did not give satisfactory purification in terms of fold purity. Hence there was a chance for further purification. Fig. 5 represents the elution chromatogram of gel filtration using Sephadex G-50. The elution chromatogram shows two peaks; first one could be because of a large molecular weight protein and second peak containing the bifunctional inhibitor. Amylase/trypsin inhibitor was found to be present between fractions 6 and 12. An overall purification of 7- and 5-fold was obtained with corresponding yield of amylase inhibitor and trypsin inhibitor being 67.4 and 50.3% respectively (Table 1). It was observed that after gel permeation chromatography the fold purification improved but the % yield was less. This could be attributed to the fact that this downstream process was carried out using three steps as compared to affinity chromatography, which had a two tier approach for purification. The yield 67.4 and 50.3% for AIU and TIU were overall yields which were compared with ammonium sulphate fraction. However the yields for the individual gel permeation chromatography step were 89 and 60.3% for AIU and TIU respectively when compared with ion exchange chromatography.

3.4. SDS-PAGE analysis

SDS-PAGE analysis of the 20–60% crude ammonium sulphate fraction, packed bed affinity, ion exchange, gel chromatography and molecular weight maker has been illustrated in Fig. 6 (photograph of SDS-PAGE developed gel). From the photograph it was seen that affinity chromatography gave a single band proving that the

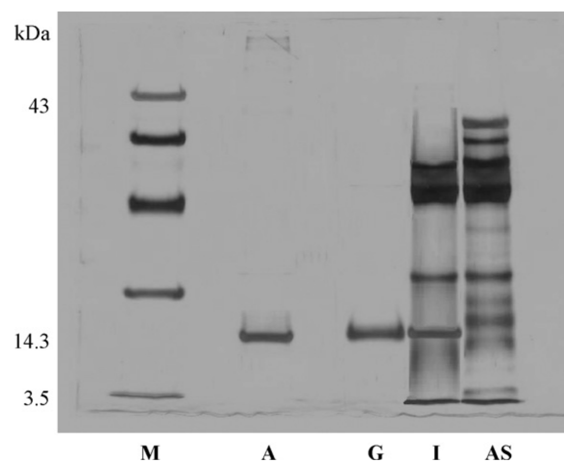

Fig. 6. SDS-PAGE of amylase/trypsin inhibitor purified fractions. M, molecular weight marker (3500–43,000 Da); A, affinity chromatography; G, ion exchange followed by gel chromatography; I, ion exchange fraction, AS, ammonium sulphate fraction (20–60%).

amylase/trypsin inhibitor was purified completely in a single step. Similarly ion exchange chromatography fraction showed multiple bands (interfering proteins) which were removed using gel permeation chromatography to give a single band. On the whole it was observed that fold purification of the fractions collected using different chromatographic separations was not large enough because they were compared with that of a partially purified ammonium sulphate fraction. However further increase in fold purity of the affinity chromatography and gel permeation chromatography fractions was not possible as these samples gave a single band in SDS-PAGE confirming the purity of the inhibitor molecule.

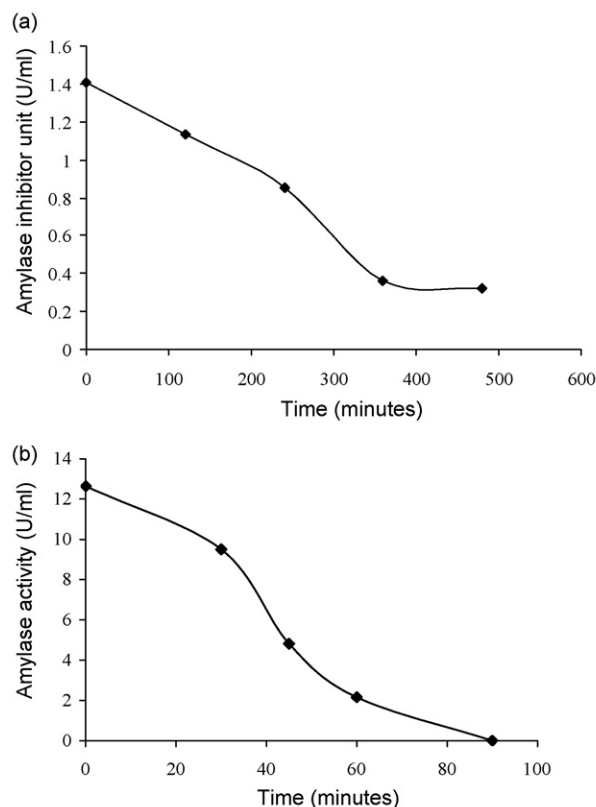

Fig. 7. (a) Immobilization of AIU on activated Seralose 6B matrix as a function of time. (b) Interaction of pancreatic amylase with immobilized amylase inhibitor as a function of time.

The molecular weight of the purified fraction was observed to be approximately 14 kDa, which matched with the reported molecular weight of ragi inhibitor protein [26]. The purified inhibitor also inhibited proteinase, which was detected using caseinolytic activity method [15]. This further established the bifunctional nature of the protein molecule.

3.5. Determination of immobilized amylase inhibitor on activated Seralose 6B matrix

The activity of amylase inhibitor in supernatant as a function of time during immobilization of amylase inhibitor (affinity ligand) on activated Seralose 6B beads is shown in Fig. 7a. It is observed that 80% of the loaded amylase inhibitor gets adsorbed in 6 h and no further increase in adsorption is seen at 8 h.

3.6. Interaction of immobilized amylase inhibitor (affinity ligand) and pancreatic amylase

The amylase activity (pancreatic amylase) in supernatant as a function of time during interaction with immobilized amylase inhibitor is shown in Fig. 7b. It is observed that this immobilized inhibitor interacts with pancreatic amylase to bring about a decrease in amylase activity in supernatant. Hence it can be concluded that the amylase/protease inhibitor from ragi holds a potential value as bioaffinity ligand for the purification of amylases.

4. Conclusions

Purification of a bifunctional amylase/trypsin inhibitor obtained from ragi (*E. coracana*) was carried out using two different downstream process approaches to observe impact of single step affinity chromatography as compared with that of two step ion exchange chromatography followed by gel chromatography. Affinity chromatography gave a fold purity of 7 and 5.3 and % yield of 82 and 72 for amylase and trypsin inhibitors respectively. On the other hand ion exchange chromatography gave a fold purity of 4.3 and 4.3 and % yield of 76 and 84 for amylase and trypsin inhibitors respectively. Gel chromatography of the ion exchange fractions gave a fold purity of 7 and 5 and % yield of 67.4 and 50.3 for amylase and trypsin inhibitors respectively.

The results proved that despite of a low fold purity, affinity chromatography of the ammonium sulphate purified fraction proved to be a better option for the purification of amylase/trypsin inhibitor because it gave almost same fold purification with higher yield of purified product in a single step as compared to the second strategy (ammonium sulphate followed by ion exchange followed by gel filtration chromatography). It is usually observed that due to availability of wide range of downstream process, it becomes difficult to make a right choice when difference in purification fold is not very high. However it is much more economical to get the same purification fold in a single step with high % yield rather than using multiple chromatography steps. So when purification is carried out to scale up for commercial application, it becomes necessary to consider technologies from yield point of view.

Amylase inhibitor obtained using affinity chromatography was further used as an affinity ligand to purify commercial pancreatic α-amylase. Interaction study of pancreatic amylase with immobilized amylase/protease inhibitor showed a reduction in α-amyalse activity. This study was primarily done as an application for observing binding efficiency of amylase/trypsin inhibitor as an affinity ligand. Purification of amylase enzyme in industries involves multiple procedures such as clarification, combined downstream process with microfiltration. If amylase enzyme can be purified using amylase/trypsin inhibitor, it will definitely help in obtaining higher yield of the enzyme with superior purity. The results demonstrated that bifunctional inhibitor from ragi (*E. coracana*) had a high selectivity for α-amyalse, and could be used for the affinity purification of commercial amylases. Further studies of bifunctional amylase/trypsin inhibitor, in order to determine interaction of inhibitors with target amylase enzyme and structure, is in progress.

Overall ragi proved to be rich as well as a cheap source of amylase/protease inhibitor. This gives us further scope for exploring other such natural and cheap sources for the development of bioaffinity ligands, which can be used in purification of enzymes and therapeutic proteins. Eventually problems faced to reduce number of unit operations as well as process economics can also be taken care of by exploiting these natural resources to their full capacity.

References

[1] K. Eric, R.M. Sandstedt, J. Am. Chem. Soc. 65 (1943) 1247.
[2] S. Murao, K. Ohyama, Agric. Biol. Chem. 39 (1975) 2271.
[3] L. Vértesy, D. Tripier, FEBS Lett. 185 (1985) 187.
[4] M. Chandrasekar, P. Parimoo, Isr. Pharm. J. 24 (1981) 60.
[5] G.P. Pal, C.B. Betzel, K.D. Jany, W. Saenger, FEBS Lett. 197 (1986) 111.
[6] S. Velankar, M.R.N. Murthy, J. Genet. 73 (1994) 43.
[7] L.F. Octávio, J.R. Daniel, R.M. Francislete, B. Carlos Jr., P.S. Carlos, F.G. Maria, Eur. J. Biochem. 267 (2000) 2166.
[8] S.T. Deeks, M. Smith, M. Holodiny, J.O. Kahn, JAMA 227 (1977) 145.
[9] H.A. Chase, J. Chromatogr. 11 (1998) 217.
[10] I. Roy, M.N. Gupta, Curr. Sci. 78 (2000) 587.
[11] B. Shivaraj, H.R. Narayana, T.N. Pattabiraman, J. Sci. Food Agric. 33 (1982) 1080.
[12] V. Buonocore, E. Poerio, F. Gramenzi, V. Silano, J. Chromatogr. 114 (1975) 109.
[13] G.L. Miller, Anal. Chem. 31 (1959) 426.
[14] R. Shainkin, Y. Birk, Biochem. Biophys. Acta 221 (1970) 502.
[15] M.L. Kakade, J.J. Rackis, J.E. McGhee, G. Puski, Cereal Chem. 51 (1974) 376.
[16] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, J. Biol. Chem. 193 (1995) 265.
[17] L. Saxena, B.K. Iyer, L. Ananthanarayan, Process Biochem. 42 (2007) 491.
[18] G.T. Hermanson, A.K. Mallia, P.K. Smith, Immobilized Affinity Ligand Techniques, Academic Press, Inc., California, 1992.
[19] R.K. Scopes, Protein Purification: Principles and Practice, second ed., Springer-Verlag, New York, 1987.
[20] B. Shivaraj, T.N. Pattabiraman, Biochem. J. 193 (1981) 29.
[21] B. Shivaraj, T.N. Pattabiraman, Ind. J. Biochem. Biophys. 17 (1980) 181.
[22] B.D. Hames, in: B.D. Hames, D. Rickwood (Eds.), Gel Electrophoresis of Proteins: A Practical Approach, IRL Press, Oxford, 1986, p. 1.
[23] L.A. Dekker, Worthington Enzyme Manual, Worthington Biochemical Corp., Freehold, NJ, 1977.
[24] J.J. Van Deemter, F.J. Zuiderweg, A. Klinkenberg, Chem. Eng. Sci. 5 (1956) 271.
[25] F.A.P. Campos, M. Richardson, FEBS Lett. 167 (1984) 221.
[26] E.J. Kruger, L. David, E.S. Clyde, in: V. Silano (Ed.), α-Amylase Inhibitors. Enzyme and Their Role in Cereal Technology, vol. 6, American Association of Cereal Chemists/St. Paul Minnesota Press Inc., USA, 1987.